

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Li et al.

Application Serial No.: to be assigned

Art Unit: to be assigned

Filed: herewith

Examiner: to be assigned

Title: Neurotransmitter Transporter

Attorney Docket No.: PF116D1C1

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir :

Prior to the prosecution of the above-captioned application, please enter the following amendments:

Amendments

In the Specification

On page 1, please insert the following paragraph and section heading after the title:

-- This application is a continuation application and claims priority under 35 U.S.C. § 120 U.S. to U.S. Application Serial No. 09/062,815, filed April 20, 1998, which is hereby incorporated by reference in its entirety, which is a divisional of and claims priority under 35 U.S.C. § 120 to U.S. Application Serial No. 08/424,424, filed April 21, 1995, which is hereby incorporated by reference in its entirety, and which is a Continuation-in-Part of PCT/US94/05363 (published in English), filed May 16, 1994, which is hereby incorporated by reference in its entirety.--

On page 1, after the first paragraph please insert as a section heading:

--Field of the Invention--

On page 4, before the first paragraph, please insert as a section heading:

--Brief Description of the Figures--

On page 4, please replace the second through fourth paragraphs with the following rewritten paragraphs:

--Figures 1A-1D show the cDNA sequence and corresponding deduced amino acid sequence of the mature NTT polypeptide. The standard one-letter abbreviations are utilized to represent the amino acid residues in the polypeptide sequence shown in Figures 1A-1D.

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figures 1A-1D, collectively, or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75713 on March 18, 1994. This deposit is a biological deposit with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, USA.

The polynucleotide of this invention was discovered in a cDNA library derived from a human fetal brain. It is structurally related to the neurotransmitter transporter family. It contains an open reading frame encoding a protein of about 727 amino acid residues. The protein exhibits the highest degree of homology to a rat neurotransmitter transporter (NTT4) with 94% identity and 96% similarity over the entire amino acid sequence.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figures 1A-1D, collectively, or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of Figures 1A-1D, collectively, or the deposited cDNA.--

Starting on page 4, please replace the paragraph bridging pages 4 and 5 with the following rewritten paragraph:

--The polynucleotide which encodes for the mature polypeptide of Figures 1A-1D, collectively, or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.--

On page 5, please replace the second through fourth full paragraph with the following rewritten paragraphs:

--The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figures 1A-1D, collectively, or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figures 1A-1D, collectively, or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figures 1A-1D, collectively, or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figures 1A-1D, collectively, or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.--

On page 6, please replace the second paragraph with the following rewritten paragraph:

--The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figures 1A-1D, collectively, or the deposited cDNA.--

On page 7, please replace the first and second full paragraphs with the following rewritten paragraphs:

--The present invention further relates to an NTT polypeptide which has the deduced amino acid sequence of Figures 1A-1D, collectively, or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figures 1A-1D, collectively, or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.--

Starting on page 7, please replace the paragraph bridging pages 7 and 8 with the following rewritten paragraph:

--The fragment, derivative or analog of the polypeptide of Figures 1A-1D, collectively, or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.--

Starting on page 23, please replace the paragraph bridging pages 23-25 with the following rewritten paragraph:

--The DNA sequence encoding for NTT, ATCC # 75713 is initially amplified using PCR oligonucleotide primers corresponding to the 5' and sequences of the processed NTT protein (minus the signal peptide sequence) and the vector sequences 3' to the NTT gene. Additional nucleotides corresponding to NTT were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence GACTAAAGCTTGGCATCAATGCCGAAGAAC (SEQ ID NO:3) contains a Hind III restriction enzyme site followed by 18 nucleotides of NTT coding sequence. The 3' sequence GAAGTTCTAGAGCAGTGGTCACAGCTCAG (SEQ ID NO:4) contains complementary sequences to Xba I site and is followed by 18 nucleotides of NTT sequence. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE-9 encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 was then digested with Hind III and Xba I. The amplified sequences were ligated into pQE-9 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform *E. coli* strain M15/rep 4 available from Qiagen under the trademark M15/rep 4 by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.⁶⁰⁰) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by

centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized NTT was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag. Hochuli, E. et al., J. Chromatography 411:177-184 (1984). NTT was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate.--

Starting on page 25, please replace the paragraph bridging pages 25 and 26 with the following rewritten paragraph:

--The DNA sequence encoding for NTT, ATCC # 75713, was constructed by PCR on the original EST cloned using two primers: the 5' primer GACTAAGATCTGCCACCATGCCGAAGAACAGCAAAGTG (SEQ ID NO:5) contains a Bgl II site followed by 21 nucleotides of NTT coding sequence starting from the initiation codon; the 3' sequence GAACTGATATCGCAGTGGTCACAGCTCAG (SEQ ID NO:6) contains complementary sequences to EcoR V site, translation stop codon, and the last 18 nucleotides of the NTT coding sequence. Therefore, the PCR product contains a Bgl II site, NTT coding sequence followed by a translation termination stop codon, and an EcoR V site. The PCR amplified DNA fragment and the vector, pcDNA1/Amp, were digested with Bgl II and EcoR V. The ligation mixture was transformed into E. coli strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant NTT, COS cells were transfected with the expression vector by DEAE-DEXTRAN method. (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the NTT HA protein was detected by radiolabelling and immunoprecipitation method. (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with ³⁵S-cysteine two days post transfection. Culture media were then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5). (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media were precipitated with a HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.--

On page 27, please replace the first paragraph with the following rewritten paragraph:

--Northern blot analysis was carried out to examine the levels of expression of NTT in human tissues. Total cellular RNA samples were isolated with RNazol™ B system (Biotech Laboratories, Inc. 6023 South Loop East, Houston, TX 77033). About 10 µg of total RNA isolated from each human tissue specified was separated on 1% agarose gel and blotted onto a nylon filter. (Sambrook, Fritsch, and Maniatis, Molecular Cloning, Cold Spring Harbor Press, (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50ng DNA fragment. The labeled DNA was purified with a Select-G-50 column. (5 Prime - 3 Prime, Inc. 5603 Arapahoe Road, Boulder, CO 80303). The filter was then hybridized with radioactive labeled full length NTT gene at 1,000,000 cpm/ml in 0.5 M NaPO₄, pH 7.4 and 7% SDS overnight at 65 degrees C. After wash twice at room temperature and twice at 60 degrees C with 0.5 x SSC, 0.1% SDS, the filter was then exposed at -70 degrees C overnight with an intensifying screen. The message RNA for NTT is abundant in brain.--

After page 27, please insert the Sequence Listing (pages 28-35). Please renumber all subsequent pages in the specification accordingly.

In the Drawings:

Informal drawings of Figures 1A-1E have been replaced with formal drawings of Figures 1A-1D.

REMARKS

The specification has been amended to add priority information, to include section headings, to insert the sequence listing identifiers where appropriate, to replace informal drawings with formal drawings, and to correct minor typographical errors. Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "Version with markings to show changes made".

No new matter has been introduced.

Applicants believe that no fee is required for this submission. However, should a fee be due, please charge such fee to Deposit Account No. 08-3425.

Respectfully submitted

Date: August 8, 2001


Joseph J. Kenny (Reg. No. 43,710)
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MMW/JJK/DS/ur

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification

On page 1, please insert the following paragraph and section heading after the title:

This application is a continuation application and claims priority under 35 U.S.C. § 120 U.S. to U.S. Application Serial No. 09/062,815, filed April 20, 1998, which is hereby incorporated by reference in its entirety, which is a divisional of and claims priority under 35 U.S.C. § 120 to U.S. Application Serial No. 08/424,424, filed April 21, 1995, which is hereby incorporated by reference in its entirety, and which is a Continuation-in-Part of PCT/US94/05363 (published in English), filed May 16, 1994, which is hereby incorporated by reference in its entirety.

On page 1, after the first paragraph please insert as a section heading:

Field of the Invention

On page 4, before the first paragraph, please insert as a section heading:

Brief Description of the Figures

On page 4, please replace the second through fourth paragraphs with the following rewritten paragraphs:

[Figure 1 shows] Figures 1A-1D show the cDNA sequence and corresponding deduced amino acid sequence of the mature NTT polypeptide. [The standard one-letter abbreviation for amino acids is used.] The standard one-letter abbreviations are utilized to represent the amino acid residues in the polypeptide sequence shown in Figures 1A-1D.

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of [Figure 1] Figures 1A-1D, collectively, or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75713 on March 18, 1994. This deposit is a biological deposit with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, USA.

The polynucleotide of this invention was discovered in a cDNA library derived from a human fetal brain. It is structurally related to the neurotransmitter transporter family. It contains an open reading frame encoding a protein of about 727 amino acid residues. The protein exhibits the highest degree of homology to a rat neurotransmitter transporter [(NT74)] (NTT4) with 94% identity and 96% similarity over the entire amino acid sequence.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in [Figure 1] Figures 1A-1D, collectively, or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of [Figure 1] Figures 1A-1D, collectively, or the deposited cDNA.

Starting on page 4, please replace the paragraph bridging pages 4 and 5 with the following rewritten paragraph:

The polynucleotide which encodes for the mature polypeptide of [Figure 1] Figures 1A-1D, collectively, or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

On page 5, please replace the second through fourth full paragraph with the following rewritten paragraphs:

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of [Figure 1] Figures 1A-1D, collectively, or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide

may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in [Figure 1] Figures 1A-1D, collectively, or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of [Figure 1] Figures 1A-1D, collectively, or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in [Figure 1] Figures 1A-1D, collectively, or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

On page 6, please replace the second paragraph with the following rewritten paragraph:

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of [Figure 1] Figures 1A-1D, collectively, or the deposited cDNA.

On page 7, please replace the first and second full paragraphs with the following rewritten paragraphs:

The present invention further relates to an NTT polypeptide which has the deduced amino acid sequence of [Figure 1] Figures 1A-1D, collectively, or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of [Figure 1] Figures 1A-1D, collectively, or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such

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tag and the RBS. The ligation mixture was then used to transform *E. coli* strain M15/rep 4 available from Qiagen under the trademark M15/rep 4 by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.⁶⁰⁰) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized NTT was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag. Hochuli, E. et al., J. Chromatography 411:177-184 (1984). NTT was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate.

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After page 27, please insert the Sequence Listing (pages 28-35). Please renumber all subsequent pages in the specification accordingly.

In the Drawings:

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